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# Ribonuclease BN: Identification and partial characterization of a new tRNA processing enzyme*

(3′ terminus/exoribonuclease/*Escherichia coli*)

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**ABSTRACT** **A new ribonuclease, RNase BN, has been identified and partially purified from a strain of *Escherichia coli* lacking RNase II and RNase D by using the artificial tRNA precursor tRNA-C-[$^{14}$C]U as substrate. This enzyme is present in *E. coli* B but absent from the tRNA processing mutant strain BN which is unable to process extraneous 3′ residues on certain phage T4-specified tRNA precursors. The properties of RNase BN clearly distinguish this enzyme from other known *E. coli* exoribonucleases. It is optimally active at pH 6.5 with 0.2 mM divalent cation and 0.2 M monovalent cation. It is most active against tRNA substrates containing nucleotide substitutions within the -C-C-A sequence and relatively inactive against other types of RNAs. This substrate specificity *in vitro* is consistent with a processing function *in vivo*. However, in contrast to the other processing enzymes whose function has been confirmed by mutation, RNase BN is an exoribonuclease. The presence of multiple RNases in *E. coli* and a strategy for their identification and separation are discussed.**

Although considerable progress has been made in elucidating the overall pathway of tRNA processing, as yet there is relatively little information available about the nucleases involved, particularly with regard to processing at the 3′ terminus (1). Earlier reports from our laboratory have described the identification, purification, and characterization of one exonuclease, RNase D, which has the *in vitro* properties expected for the processing nuclease that would remove extra residues following the -C-C-A sequence in *Escherichia coli* tRNA precursors (2–6). However, more recent experiments with *E. coli* strains deficient in RNase D have raised uncertainties about its actual physiological role (7, 8).

The existence of a second tRNA processing exoribonuclease has been suggested by the isolation of an *E. coli* mutant strain, termed BN (9). This strain is affected in its ability to process the 3′ terminus of certain tRNA precursors synthesized after bacteriophage T4 infection (10), although growth of the host is not impaired (unpublished data). The affected T4 precursors lack all or part of the usual -C-C-A sequence and require removal of extraneous nucleotides prior to -C-C-A completion. Schmidt and McClain suggested that this processing defect was due to the loss of a specific enzyme, termed "BN ribonuclease" (11). Although the enzyme described by these workers was not characterized in detail, the properties that were reported were identical to those now attributed to RNase D (5), rather than to a new enzyme, raising questions about the existence of a distinct BN ribonuclease. However, further genetic and biochemical studies in our laboratory have shown that the mutation in strain BN is distinct from RNase D (8, 12), encouraging us to search further for the enzyme affected by the BN mutation.

Establishing the existence of RNase BN has been hampered by the large amounts of RNase II and RNase D generally found in extracts, which tend to mask other less-active enzymes under usual conditions of assay. However, using mutant *E. coli* strains deficient in RNase II and RNase D, we have now been able to detect an exoribonuclease that is present in *E. coli* B but absent from *E. coli* BN. In this paper we describe the identification of this new processing enzyme and present an initial description of its properties.

## MATERIALS AND METHODS

**Bacterial Strains.** Wild type *E. coli* B and its derivative, strain BN, were obtained from William McClain (University of Wisconsin). Strain BN does not support the growth of T4 phages which are dependent on the suppressor function of T4 serine tRNA (9). RNase II$^-$ (*rnb*) and RNase D$^{ts}$ (*rnd*) derivatives of these strains were constructed by P1-mediated transduction as described (7) for derivatization of other *E. coli* strains. The strains were first made trp$^-$ by using the Tn*10* in the *trp* locus harbored by strain NK5151. Transfer of the *rnb* and *rnd* mutations from their resident K-12 strains to strain B proceeded with much lower transduction efficiency than usual due to restriction of phage P1. However, once the B *rnb,rnd* derivative was constructed, the mutant *rnb* and *rnd* loci were transferred directly from strain B to BN, rather than proceeding from the K-12 strains. The *rnb,rnd* derivatives of strains B and BN contained <2% of normal levels of RNase II and, after heating at 45°C, contained <20% of normal RNase D activity. *E. coli* strains S296 and S296-680 (13) were obtained from David Schlessinger (Washington University). Strain S296 is the original isolate harboring the *rnb* mutation (13), and S296-680 is a derivative deficient in another unknown exoribonuclease (14).

**Substrates.** [$^3$H]poly(A) and nonradioactive poly(A) were purchased from Miles. Phosphodiesterase-treated [$^{32}$P]tRNA (dtRNA) and [$^{32}$P]rRNA were prepared as described (2, 8). tRNA-C-[$^{14}$C]U, tRNA-C-[$^{14}$C]A, tRNA-C-[$^{14}$C]C, tRNA-[$^{14}$C]U, and tRNA-C-C-A-[$^{14}$C]Cn were synthesized by using liver tRNA nucleotidyltransferase as reported (2, 15).

**Other Materials.** RNase D was purified as reported (5). Bacterial alkaline phosphatase was obtained from Sigma. Ultrogel AcA44 was purchased from LKB. All salts were reagent grade.

**Preparation of Extracts.** The high-speed supernatant (S100) fractions from *E. coli* were prepared after rupture of the cells in an Aminco French press as described (5). The S100 fractions were concentrated 5-fold in an Amicon ultrafiltration apparatus prior to gel filtration. RNase D activity in extracts of strains B*rnb,rnd* and BN*rnb,rnd* was eliminated by heating at 45°C for 15 min.

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Abbreviation: dtRNA, snake venom phosphodiesterase-treated tRNA.

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**Enzyme Assays.** Nuclease activity against the various substrates was measured by determination of acid-soluble radioactivity as described (2, 3). Reaction mixtures for the assays in Fig. 1 against [$^3$H]poly(A), [$^{32}$P]dtRNA, and tRNA-C-[$^{14}$C]U have been described (2, 3). For determination of RNase I activity shown in Fig. 2, the 100-$\mu$l reaction mixtures contained: 20 mM Tris·HCl at pH 7.5, 2 mM EDTA, 100 mM KCl, and 40 $\mu$g of [$^{32}$P]dtRNA. Assays for activity against [$^{32}$P]rRNA in Fig. 2 contained (in 100 $\mu$l): 20 mM Hepes at pH 6.5, 0.2 mM $CoCl_2$, 300 mM KCl, and 40 $\mu$g of [$^{32}$P]rRNA. Standard assays for RNase BN in Fig. 2 and Table 2 contained (in 100 $\mu$l): 20 mM Hepes at pH 6.5, 0.2 mM $CoCl_2$, 200 mM KCl, and 19 $\mu$g of tRNA-C-[$^{14}$C]U or other tRNA substrates.

## RESULTS

**Evidence for the Existence of a RNase BN.** Because the earlier work on a putative RNase BN (11) did not clearly distinguish this activity from RNase D (6), we thought it important first to demonstrate the existence of such an enzyme. In our initial studies we tested for an activity that would be present in *E. coli* B but absent from *E. coli* BN and that could remove [$^{14}$C]-UMP from the artificial tRNA precursor tRNA-C-[$^{14}$C]U. However, due to the presence of high levels of RNase II and RNase D, which also can act on this substrate, we could not show convincingly by biochemical procedures that an additional enzyme was present in *E. coli* B. On the other hand, using genetic procedures, we were able to demonstrate that the tRNA processing defect in strain BN was not due to a mutation in RNase II or RNase D (12) and that the BN mutation did not affect a factor that influenced RNase D activity (8). In addition, we could not detect any differences between RNase D preparations extensively purified from strains B and BN (unpublished data).

Direct biochemical evidence for a distinct RNase BN was obtained by heat-inactivation studies of S100 fractions from strains B and BN (Fig. 1). At 55°C, RNase II and RNase D were rapidly inactivated as shown by the loss of poly(A) and dtRNA hydrolytic activities. Each of the two activities were lost to the same degree (≥90%) in strains B and BN. In contrast, loss of tRNA-C-U hydrolytic activity reached a plateau at about 50% and 25% of the original activities in extracts of strains B and BN, respectively. The findings that heat inactivation with tRNA-C-U as substrate differed in the two strains and that it reached a plateau considerably above that seen with poly(A) and dtRNA indicated that strain B contained an activity, missing in BN, that hydrolyzed tRNA-C-U and amounted to about 25% of the total activity against this substrate under these assay conditions. In addition, the observation that strain BN still retained about 20–25% of its original activity, even when RNase II and RNase D were inactivated, suggested the presence of yet another enzyme that could act on tRNA-C-[$^{14}$C]U, distinct from any of the aforementioned nucleases (see below).

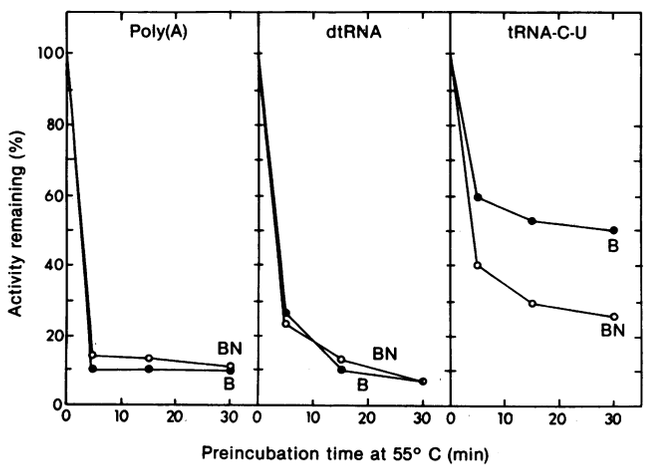


FIG. 1. Effect of heat inactivation on RNase activities in extracts of strains B and BN. High-speed supernatant fractions from strains B and BN were incubated at 55°C for the indicated times prior to assay for activity against [$^3$H]poly(A), [$^{32}$P]dtRNA, or tRNA-C-[$^{14}$C]U. Activities are expressed relative to an unheated sample taken as 100%.

**Identification of RNase BN.** Because it was clear that RNase II and RNase D interfered with the detection of the RNase BN, derivatives of strains B and BN were constructed that contained mutations in the *rnb* and *rnd* genes (7, 16). These strains were devoid of RNase II activity and contained a temperature-sensitive RNase D that could be inactivated readily by incubation at 45°C. Gel filtration (on Ultrogel Aca44) of heated extracts of these derivative strains revealed a major peak of activity against tRNA-C-[$^{14}$C]U that was present in strain B but completely missing in strain BN (Fig. 2). This enzyme was about the same size as RNase II (4) and alkaline phosphatase. If it is assumed to be a globular protein, it has a molecular weight of about 80,000. A second peak of activity against tRNA-C-[$^{14}$C]U was also observed in the low molecular weight region of the column but was present in both strains B and BN. Studies of the properties of this activity, and the use of appropriate mutants, indicated that this activity was largely due to RNase I (unpublished data). The presence of RNase I would explain the residual heat-resistant activity against tRNA-C-[$^{14}$C]U seen in strain BN (Fig. 1). These results conclusively identify RNase BN in *E. coli* strain

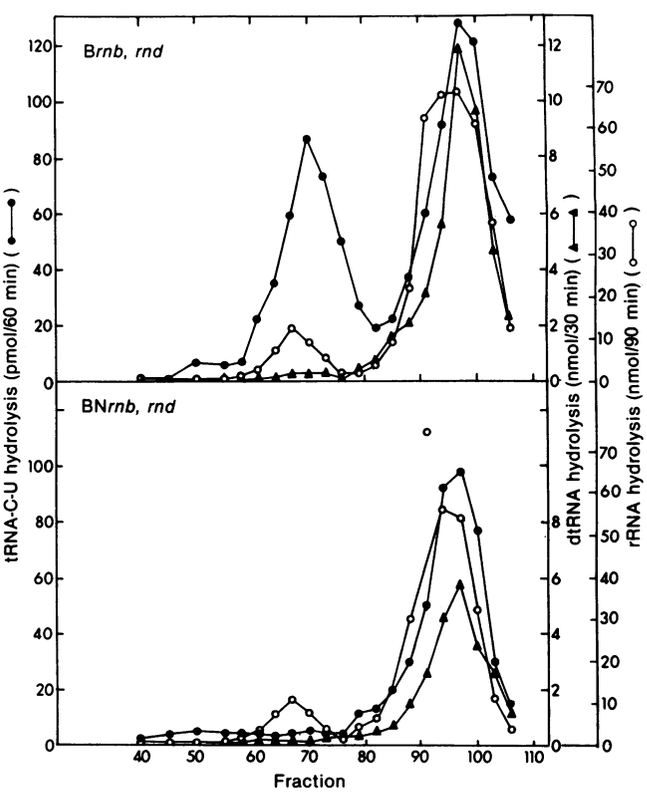


FIG. 2. Gel filtration of high-speed supernatant fractions from strains B*rnb,rnd* and BN*rnb,rnd*. One-milliliter aliquots of the concentrated, heated S100 fractions from each strain were chromatographed on a column of Ultrogel AcA44 (1.5 × 94 cm). The column was equilibrated and run in 20 mM Tris·HCl, pH 7.5/5 mM $MgCl_2$/0.1 mM EDTA/0.1 mM dithiothreitol/0.1 mM phenylmethylsulfonyl fluoride/1 M KCl/10% (vol/vol) glycerol. Fractions (1.15 ml) were collected at a flow rate of 6 ml/hr. Aliquots were assayed for activity against tRNA-C-[$^{14}$C]U, [$^{32}$P]rRNA, and [$^{32}$P]dtRNA in the presence of EDTA.

B and demonstrate its absence in the tRNA processing mutant strain BN.

Another feature apparent from Fig. 2 is the presence of an additional nuclease that elutes in the region of RNase BN. However, this nuclease is present in both strains B and BN and is active against rRNA. Inasmuch as strain BN is devoid of activity against tRNA-C-[$^{14}$C]U in this region of the column, this contaminating RNase did not interfere with the characterization of RNase BN. Studies with S296-680 (13), a mutant *E. coli* strain, revealed that the contaminating RNase was absent but that RNase BN was present in extracts of this strain (unpublished data). Strain S296-680 also is able to plate $psu_1^+$-amber T4 phage normally (unpublished data), whereas strain BN cannot (9). Thus, these data indicate that the RNase defined by the mutation in S296-680 is distinct from RNase BN and presumably represents the exoribonuclease previously identified by Schlessinger and co-workers (14).

**Properties of RNase BN.** By using tRNA-C-[$^{14}$C]U as substrate, the catalytic properties of the partially purified RNase BN from Ultrogel AcA44 were determined. With this substrate the pH optimum of the enzyme was found to be at pH 6.5 in 0.02 M Hepes. About 90% of optimal activity was obtained at pH 6.0 and 7.0, and about 70% of optimal activity was found at pH 8.0.

RNase BN was stimulated by both divalent and monovalent cations. The optimal divalent cation concentration was about 0.2 mM, with $Co^{2+}$ being about twice as effective as $Mg^{2+}$. At 0.2 mM $Co^{2+}$ the enzyme was stimulated about 3-fold by the addition of KCl. The optimal KCl concentration for stimulation was 0.2 M with about 70% of maximal activity being obtained at 0.6 M.

All the radioactivity released from tRNA-C-[$^{14}$C]U by RNase BN was in the form of the mononucleotide UMP because it could be completely converted to uridine by treatment with alkaline phosphatase (Table 1). In this regard, RNase BN behaved identically to RNase D, indicating that RNase BN also is an exoribonuclease.

A preliminary survey of the substrate specificity of RNase BN was also carried out. Because the partially purified RNase BN was still contaminated with another RNase, it was necessary to compare activities against various substrates by using equivalent fractions from strains B*rnb,rnd* and BN*rnb,rnd* from the columns as shown in Fig. 2. Substrates that were preferentially hydrolyzed by the strain B fraction were assumed to be acted upon by RNase BN, whereas those hydrolyzed similarly by both fractions were assumed not to be substrates for this RNase. tRNA-[$^{14}$C]U, tRNA-C-[$^{14}$C]U, tRNA-C-[$^{14}$C]A were hydrolyzed efficiently by RNase BN (Table 2). In contrast, [$^{3}$H]poly(A), [$^{32}$P]-rRNA (from Fig. 2), tRNA-C-[$^{14}$C]C, [$^{32}$P]dtRNA, and tRNA-C-C-A-[$^{14}$C]Cn were poor substrates for this enzyme under these assay conditions. Interestingly, the latter two tRNA derivatives are excellent substrates for RNase D (4). These results suggest that RNase BN is highly specific for tRNA substrates containing other nucleotides in place of the usual -C-C-A sequence at the 3′ terminus.

Table 1. Mode of hydrolysis of tRNA-C-[$^{14}$C]U by RNase BN and RNase D

| | Radioactivity released as | | | |
|---|---|---|---|---|
| | Nucleoside | | Nucleotide | |
| Enzyme | cpm | % | cpm | % |
| RNase BN | 1,589 | 96 | 72 | 4 |
| RNase D | 417 | 85 | 72 | 15 |

The acid-soluble fraction from an assay with RNase BN or RNase D was extracted four times with ether to remove trichloroacetic acid. The samples were then treated with 0.5 unit of bacterial alkaline phosphatase in 10 mM $NH_4HCO_3$, pH 8.8/5 mM $MgCl_2$ at 45°C for 30 min. The samples were neutralized and passed through a 1-ml Dowex-1-Cl column. Radioactive nucleoside (uridine) was eluted with $H_2O$, and nucleotide material was eluted with 0.1 M HCl. The total radioactivity released in each form is presented as well as the percentage distribution.

Table 2. Substrate specificity of RNase BN

| | Nucleotides released, pmol/60 min | | |
|---|---|---|---|
| Substrate | Strain B | Strain BN | Ratio, B/BN |
| tRNA-C-[$^{14}$C]U | 67 | 2.3 | 29 |
| tRNA-C-[$^{14}$C]A | 46 | 4.2 | 11 |
| tRNA-C-[$^{14}$C]C | 2.1 | <2.0 | >1.1 |
| tRNA-[$^{14}$C]U | 120 | 7.6 | 16 |
| tRNA-C-C-A-[$^{14}$C]Cn | 6 | 3 | 2.0 |
| [$^{32}$P]dtRNA | 300 | 230 | 1.3 |
| [$^{3}$H]Poly(A) | 6,300 | 6,000 | 1.1 |

Assays were carried out for 60 min at 37°C with 19 μg of each tRNA substrate (≈20 cpm/pmol) and 20 μl of peak enzyme fractions from the column as shown in Fig. 2.

## DISCUSSION

The studies presented in this paper demonstrate the existence of a new *E. coli* RNase termed "RNase BN." Our ability to identify and to characterize this enzyme was made possible by the construction of *E. coli* strains lacking RNase II and most RNase D activities. Because these latter two nucleases also could act on the artificial tRNA precursor tRNA-C-[$^{14}$C]U, specific assay of RNase BN in crude extracts was not possible. In addition, the similarity in chromatographic properties of many of the *E. coli* RNases makes separation and conclusive identification of any new processing nuclease a difficult task. However, once the major interfering activities, RNases II and D, were removed, optimization of RNase BN assay conditions was accomplished and conclusive identification was possible.

The approach taken here for identifying RNase BN indicates the type of strategy that appears to be most effective in unraveling the complexity of the multiple RNases present in a cell. These include the use of assays across columns with numerous RNA substrates, of which one mimics the natural precursor, plus the appropriate use of mutants when possible. Using this approach, we have also shown the presence of an additional RNase that acts effectively on rRNA and have clarified the contribution of RNase I to tRNA-C-[$^{14}$C]U hydrolysis in crude extracts. As the catalogue of characterized RNases in a given cell increases, subsequent identification of new enzymes should be made easier.

The catalytic properties of RNase BN, as well as the existence of a specific mutant, clearly differentiate this enzyme from other *E. coli* exoribonucleases previously described. RNase BN is optimally active at a lower pH, a lower divalent cation concentration, and a higher monovalent cation concentration than either RNase II or RNase D. RNase BN is inactive, or poorly active, against various substrates such as poly(A), dtRNA, tRNA-C-C-A-Cn, and rRNA which are effective substrates for other RNases. RNase II can act on all these RNAs (2–4), RNase D can hydrolyze dtRNA and tRNA-C-C-A-Cn (2–4), and the exoribonuclease described by Gupta *et al.* (14) is active against poly(A) and rRNA. In contrast, RNase BN is active against tRNA-C-U, a relatively poor substrate for both RNase II and RNase D (3). Most importantly, each of these RNases also can be distinguished by mutations that selectively inactivate only one of the four enzymes at a time (7, 8, 13, 14). Thus, *E. coli* appears to contain at least four distinct hydrolytic exoribonucleases.

It is likely that RNase BN is a tRNA processing enzyme. McClain and co-workers (10) have shown that strain BN does not process precursors for T4-specified $tRNA^{Ser}$, $tRNA^{Pro}$, and $tRNA^{Ile}$, whereas precursors to five other tRNAs are processed normally. Each of the three tRNA precursors that are not processed properly contain residues other than the normal -C-C-A sequence at their 3′ terminus, and these residues are not removed in the BN mutant, which lacks RNase BN. The five other precursors that are processed correctly in strain BN either already contain the -C-C-A sequence followed by additional residues or do not require exoribonuclease action for -C-C-A synthesis (17). Possibly, these five precursors, which are like the host precursors, utilize RNase D for 3′ terminal processing. Although revertants of strain BN that have simultaneously regained RNase BN and tRNA processing activity have not yet been obtained, the *in vitro* specificity of RNase BN fits closely with its presumed *in vivo* role. It is most active on the substrates tRNA-U, tRNA-C-U, and tRNA-C-A, all of which contain an extraneous residue at their 3′ terminus. Interestingly, tRNA-C-C, which would be an intermediate in the synthesis of the mature -C-C-A sequence, is relatively resistant to RNase BN. Thus, it is reasonably certain that RNase BN serves to process the 3′ terminus of some T4 tRNA precursors *in vivo*. However, because all *E. coli* tRNA precursors and tRNA genes studied to date contain the -C-C-A sequence (18), the function of RNase BN in uninfected cells remains to be determined.

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